Exhibit A

JOURNAL OF VIROLOGY, Apr. 1997, p. 2921–2927
0022-538X/97/$04.00+0

Vol. 71, No. 4

Pre- and Postexposure Protection by Passive Immunoglobulin but No Enhancement of Infection with a Flavivirus in a Mouse Model

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Received 27 September 1996/Accepted 31 December 1996

Antibody-dependent enhancement of flavivirus infection, which except for dengue virus is without clear proof in vivo, is still under debate. Recently, postexposure immunoglobulin prophylaxis against tick-borne encephalitis virus, a flavivirus, was claimed to possibly have worsened the outcome of infection due to antibody-dependent enhancement. In the present study, antibody-dependent enhancement and pre- or postexposure protection by passive administration of tick-borne encephalitis virus immunoglobulin were evaluated in a mouse model. Preexposure treatment with homologous murine or heterologous human immunoglobulin provided complete protection against lethal challenge with tick-borne encephalitis virus. For postexposure treatment with antibody, the degree of protection correlated with the amount of immunoglobulin administered and was inversely related to the time interval between infection and treatment. Indications of enhancement of infection would have been increased lethality or reduced mean survival time, but neither was observed under the conditions used in our experiments despite the broad range of immunoglobulin and virus challenge doses applied. In contrast to these in vivo results, antibody-dependent enhancement of tick-borne encephalitis virus infection of murine peritoneal macrophages was readily demonstrable in vitro. Thus, antibody-dependent enhancement of viral infection in vitro does not necessarily predict enhancement in vivo.

The flavivirus genus of the *Flaviviridae* family comprises a number of important human pathogens causing diseases such as yellow fever, Japanese encephalitis, dengue hemorrhagic fever and shock, Saint Louis encephalitis, and tick-borne encephalitis (TBE) (31). Good evidence for antibody-dependent enhancement (ADE) as a pathogenic factor in vivo has been provided for dengue virus (10) by studies of disease in humans as well as by animal studies, but the role of ADE in infection with other flaviviruses is still a matter of debate. TBE virus (TBEV), which is endemic in parts of Europe and Asia, is one of the most pathogenic flaviviruses for humans. Active immunization with a safe and efficacious whole-killed virus vaccine (12, 21) is state of the art in several countries, e.g., Austria, but for those not vaccinated when bitten by a tick in an area of endemicity, passive immunization with TBEV immunoglobulin (Ig) is also available (22). In the context of active and passive immunization strategies, however, ADE of infection is always a concern. For TBEV, ADE has been demonstrated in vitro (17, 27), and the possibility of ADE in vivo has been raised (16). Furthermore, presently available results from human studies in support of passive protection by antibodies (20, 22) have been questioned (1). As it has been suggested that only 1 in 1,000 tick bites is followed by disease in an area of endemicity (22), the number of volunteers required to address the question in a controlled clinical trial would make such investigations hardly feasible. Furthermore, in regions of endemicity where Ig prevention after tick bite is state of the art for unvaccinated people, ethical problems would be difficult to solve.

Experimental infection of mice has provided a useful model for studying immune mechanisms in relation to infection with TBEV, although data characterizing the model are scarce and not fully congruent (15, 18, 19). Nevertheless, transfer of monoclonal antibodies recognizing the TBEV surface glycoprotein E (11, 25, 28) or antiserum of the respective specificity (13) into mice led to the current understanding that protection against TBE after TBEV challenge is mediated by antivirion antibodies.

In the present study, the efficacy of pre- and postexposure protection by a polyclonal human TBEV Ig preparation was tested in this mouse model, and the question of an eventual ADE of TBEV infection in vivo was addressed. To control for the relevance of the results obtained with heterologous human Ig in a murine model, parallel experiments were also performed with homologous murine TBEV antibodies.

MATERIALS AND METHODS

Animals. Female BALB/c mice (Charles River Wiga, Sulzfeld, Germany) (15 to 17 g, body weight) were used throughout the experiments.

Virus. TBEV, strain Neudörfl, a flavivirus (31), was kindly donated by P. Noel Barrett (Biomedical Research Center, IMMUNO AG, Orth/Donau, Austria). TBEV was propagated in Vero cells (CCL 81; American Type Culture Collection, Rockville, Md.) and purified from culture supernatant by ultrafiltration and subsequent ultracentrifugation. The resulting virus pellet was resuspended in phosphate-buffered saline (PBS).

Virus titration. TBEV titers were determined by plaque assay (6), with minor modifications. Briefly, porcine stable (PS) cell monolayers were incubated with serial 10-fold dilutions of samples, and after 4 days the cells were formaldehyde fixed and stained with crystal violet, and the plaques were counted. Virus titers are expressed as PFU per ml of sample.

To quantify virus titers in blood or brain of TBEV-inoculated animals, blood was aseptically collected by cardiac puncture of ether-anesthetized animals or brains were aseptically removed after cervical dislocation from two identically treated animals per sample. The two brains were, after addition of 1.8 ml of PBS, homogenized through a steel mesh, or the blood of two animals (approximately 1 ml) was diluted by addition of an equal volume of PBS. The resulting samples were then subjected to three freeze-thaw cycles. Centrifugation-clarified homogenates were finally tested by plaque assay.

For high-sensitivity detection of infectious TBEV in samples, blood or brain samples were prepared as before, and one-third of the resulting specimen was

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immediately transferred intraperitoneally (i.p.) into each of three recipient animals per sample. Survival of recipient animals was monitored for 28 days.

The detection limits for the plaque assay (approximately 1,000 PFU/organ) and the sample transfer (approximately 10 PFU/organ) were determined by spiking samples with TBEV (data not shown).

Infection of mice. The mice were experimentally inoculated with TBEV by i.p. or subcutaneous (s.c.) injection of 0.2 ml of TBEV stock, diluted to contain 1,000 PFU as determined by plaque assay. Survival of TBEV-challenged animals was recorded for 28 days after infection or until no further deaths occurred for 7 consecutive days.

TBEV antibodies. In the current study, either human or murine TBEV antibodies were used. For human TBEV antibodies, a commercially available and clinically used preparation of human TBEV Ig, FSME BULIN (IMMUNO AG), was utilized; it is prepared from plasma of donors immunized with a whole-killed TBEV vaccine (FSME-IMMUN Inject; IMMUNO AG) prepared from TBEV strain Neudörfl. The preparation contains 90 to 153 mg of gamma globulin per ml and has TBEV titers as follows (given are titer ± standard error of the mean [SEM]; *n* is the number of determinations): hemagglutination inhibition (HAI) titer, 1:1,100 ± 60 ($n = 16$); neutralization titer (NT), 1:4,406 ± 518 ($n = 9$). The recommended doses for treatment of humans are 0.05 ml/kg for preexposure prophylaxis and 0.1 ml/kg for postexposure prophylaxis until up to 48 h after suspected exposure or 0.2 ml/kg for between 48 and 96 h after exposure.

For murine TBEV antibodies, mice were s.c. immunized with 0.2 ml of a 1:10-diluted whole-killed TBEV vaccine (FSME-IMMUN Inject; IMMUNO AG), three times at three-week intervals. The hyperimmune serum obtained was heat inactivated and further used as the source of TBEV antibodies. The respective TBEV titers were the following (titer ± SEM; *n* is the number of determinations): HAI titer, 1:747 ± 107 ($n = 6$); NT, 1:983 ± 126 ($n = 6$).

Passive protection of mice. For passive protection, the mice were administered TBEV antibodies 2 h before TBEV challenge, i.e., pre-exposure prophylaxis, or at various intervals thereafter, i.e., postexposure prophylaxis. The mice were treated with antibodies by s.c. injection of 0.2 ml of either human TBEV Ig or mouse hyperimmune serum, both diluted as indicated with PBS. When mice were s.c. treated with both TBEV and Ig, injections were performed on opposite sides of the animal.

ELISA of TBEV antibodies. Enzyme-linked immunosorbent assay (ELISA) titers of human TBEV IgG in murine serum were determined with a commercially available reagent kit (IMMUNOZYM FSME IgG; IMMUNO AG) according to the manufacturer's recommendations. Briefly, TBEV-specific antibodies were captured from sera by their binding to plastic wells coated with inactivated TBEV antigen. Bound antibodies were then incubated with an anti-human IgG-peroxidase conjugate, and the reaction was visualized by color development with tetramethylbenzidine and quantified by reading the optical density at 450 nm. From a standard curve obtained by assay of included standards, the titer of a given sample in Vienna International Units can be calculated.

NT. Serial twofold dilutions of murine sera or human Ig preparations were incubated for 2.5 h at 25°C with approximately 100 tissue culture infective doses of TBEV before replicates of the mixtures were incubated for 7 days on TBEV-susceptible Vero cell monolayers. In the resulting supernatants, TBEV antigen, whose presence is indicative of TBEV replication, was then tested by ELISA, and the NT, i.e., the sample dilution resulting in virus neutralization in 50% of the replicates, was calculated.

HAI titer. Serial twofold dilutions of murine sera or human Ig preparations were incubated for 1 h at 25°C with TBEV. Triplicates of the resulting sample were then incubated for 3 h at 25°C with goose erythrocytes to allow antibody-free TBEV to induce hemagglutination. The HAI titer is the reciprocal of the highest sample dilution resulting in complete inhibition of hemagglutination.

In vitro ADE of TBEV infection. Resident macrophages were prepared from the peritoneal exudate of BALB/c mice by allowing 2×10^6 peritoneal exudate cells to adhere to 24-well cluster plates (Costar Corp., Cambridge, Mass.). After 2 h, nonadherent cells were removed by washing with warm PBS, and macrophages were cultured further in Dulbecco's modified Eagle's medium (25 mM HEPES and 4 g of glucose per liter; GIBCO BRL, Gaithersburg, Md.), with additional 5% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah), 2 mM glutamine, 100 μg of streptomycin per ml, and 100 U of penicillin G per ml (all from JRH Biosciences, Lenexa, Kans.). After overnight culture, TBEV antibodies were added to reach final concentrations as shown in Fig. 5, followed by TBEV at 5 PFU per cell. After incubation for 1 h, antibodies and nonadsorbed virus were removed by washing the cells with warm PBS before they were again supplemented with complete medium. Culture supernatants were collected 24 h after infection, and TBEV titers were determined by plaque assay as described.

RESULTS

TBEV infection of mice. Experimental infection of mice is, although poorly defined, a useful model for studying immune mechanisms relevant to protection from TBEV infection. In the model, TBEV infection was performed by either i.p. injection (11, 13, 25) or, anatomically more similar to natural infection via tick bite, s.c. injection (15, 28). When i.p. and s.c. TBEV challenges of mice were compared, the 50% lethal dose (LD_{50}) after i.p. infection required less virus compared to that for s.c. infection (LD_{50} [i.p. challenge], 4 PFU/mouse; LD_{50} [s.c. challenge], 14 PFU/mouse; calculated with sigmoidal dose-response curve; chi-square test, $P < 0.0001$). Mean survival times (MST) after challenge with 1,000 PFU per mouse via either route, however, were comparable (mean ± SEM: 240 ± 14 h for i.p. challenge; 236 ± 11 h for s.c. challenge; Student's *t* test, $P = 0.457$), and this challenge dose was further used for passive protection experiments except where otherwise indicated.

Development of viremia and TBEV infection of the brain were subsequently determined in blood or brain specimens obtained at different times after either s.c. or i.p. infection of mice. Plaque titration was employed to yield quantitative results, whereas sample transfer into naive recipients provided more sensitive yet semiquantitative results.

By titration on PS cells (Fig. 1, upper panels), viremia became detectable at 24 h after either i.p. (upper left panel) or s.c. (upper right panel) infection and lasted for a further 2 days before levels of TBEV in the blood dropped to below the detection limit. Detectable levels of TBEV in the brain could be verified approximately 6 days after virus inoculation; the titers then increased dramatically and remained high until death of the animals. By transfer of samples into naive recipients (Fig. 1, lower panels), viremia was detectable 24 h after infection of mice and throughout infection. While the results of the experiments described so far were similar for i.p. and s.c. infections, transfer of brain samples revealed a delay of TBEV replication in the brain of approximately 1 day after s.c. infection compared with that seen after i.p. infection of mice: in situ TBEV replication as opposed to contaminations of brain samples with viremic blood (2) started from approximately 72 h after i.p. challenge and 96 h after s.c. challenge.

Treatment of mice with human TBEV Ig. To characterize the fate of human TBEV Ig after administration to mice, TBEV ELISA, NT, and HAI titers were determined with mouse sera obtained at different times after administration of human Ig (Fig. 2). Titers of binding and functional antibodies reached their maximum between 1 and 2 days after Ig treatment, before beginning to decline approximately in parallel. With approximately 78 to 80 ml of blood per kg for a mouse (4a), i.e., approximately 1.3 ml, comparison of the NT and HAI titers of the Ig preparation with those reached in the blood of treated mice resulted in a theoretical in vivo recovery of 70 to 110%.

Passive protection by human or murine TBEV Ig. To determine the potency of preexposure protection of either the human TBEV Ig preparation or murine TBEV hyperimmune serum, 0.2 ml of various antibody dilutions from either source were administered s.c. to mice 2 h before they were challenged i.p. with 1,000 PFU of TBEV. Due to higher TBEV antibody titers (NT, 1:4,406 vs. 1:983; HAI, 1:1,100 vs. 1:747, respectively) the human Ig preparation provided protection at a higher dilution than the murine serum (effective dose providing 50% survival: 1:135 for human Ig and 1:11 for murine hyperimmune serum). Blood or brain samples of protected mice given human Ig at a dose of 0.2 ml of a 1:10 dilution s.c. 2 h prior to challenge with 1,000 PFU i.p. were tested for the presence of infectious TBEV. The protection appears to be protection from infection, as with the very sensitive method of sample transfer, TBEV could never be detected in blood or brain. To characterize the decline of protection by TBEV antibodies, mice were treated s.c. with either human heterologous (1:10) or murine homologous (1:3) Ig and were chal-

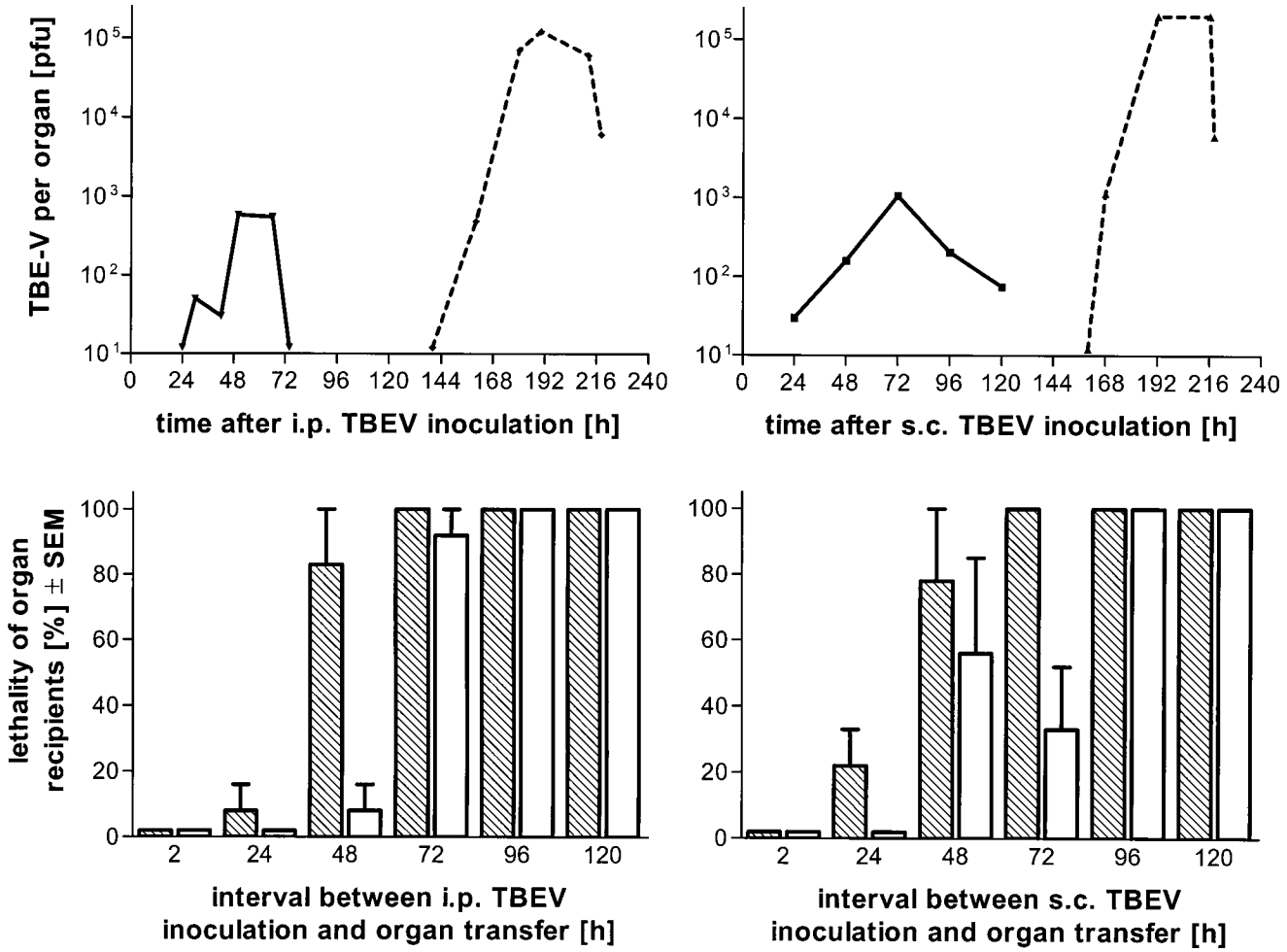

FIG. 1. Mice were infected with 1,000 PFU of TBEV by i.p. (left panels) or s.c. (right panels) injection. At the indicated times after infection, blood or brain samples were prepared from two donor animals per sample as described in Materials and Methods, and either TBEV titers were determined by plaque assay (upper panels) or aliquots of one-third of the samples were transferred i.p. into each mouse in groups of three naive recipients for sensitive detection of TBEV (lower panels). Data given are in PFU per organ. (Upper panels) Levels of virus in the blood (solid lines) or the brain (dashed lines). Shown are results from one representative experiment of three experiments. (Lower panels) Lethality of groups of naive recipients after transfer of the respective organs; blood (hatched bars) or brain (open bars). Shown are results from three or more experiments (means ± SEM).

lenged at various intervals thereafter with 1,000 PFU of TBEV i.p. The observed decline in protection paralleled the decline in serum TBEV titers (data not shown) and, due to immune elimination, was faster in the heterologous than in the homologous system, as follows (survivors [percent] ± SEM): 2 h after Ig treatment, human Ig, 97.5 ± 2.5; murine Ig, 83 ± 17; 28 days after Ig treatment, human Ig, 10 ± 7; murine Ig, 45 ± 5. Thus, at 28 days after Ig treatment the proportion of mice still passively protected against TBEV challenge had dropped to 10 and 54% of the initial values for human and murine Igs respectively.

For further experiments, a highly protective dose (1:10 for human Ig and 1:3 for murine Ig) (Fig. 3) and one providing only suboptimal protection when applied preexposure (1:100 and 1:30, respectively) (Fig. 3) were used, the latter to favor detection of ADE.

As can be seen from Fig. 3, preexposure treatment with either heterologous human (left panels) or homologous murine (right panels) Ig was able to protect mice against otherwise lethal i.p. (upper panels) or s.c. (lower panels) challenge with 1,000 PFU of TBEV per animal. Overall, the protection achieved by passive Ig was dose dependent, i.e., higher concentrations provided better protection than lower concentrations of the same Ig preparation. For postexposure prophylaxis, the protective effect decreased with increasing time interval between Ig treatment and TBEV challenge. Nevertheless, even when late Ig treatment protected only a small proportion of TBEV-inoculated animals or was not protective, MST of groups of Ig-treated and TBEV-infected mice versus TBEV-infected controls was always prolonged or equal but never reduced (Fig. 4). As in vitro ADE is best demonstrable at low ratios of virus-to-target cells and subneutralizing concentrations of antibodies, further experiments were performed with challenge doses down to 10 PFU per animal, i.e., approximately an LD_{50} and Ig dilutions as low as 1:3,000. As before, however, Ig treatment resulted in an increase in survival rate or MST of TBEV-infected animals compared to that for infected controls without Ig treatment or had no effect, but it never caused ADE of infection (data not shown). Passive protection against s.c. TBEV challenge was considerably more potent than that against i.p. challenge and could also be achieved for

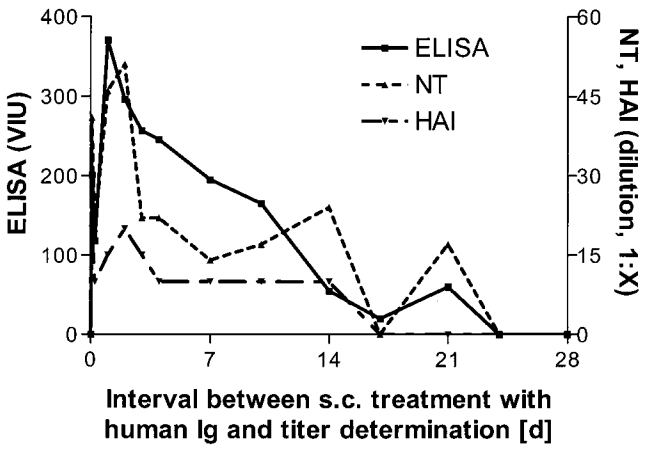

FIG. 2. Mice were s.c. injected with 0.2 ml of a human TBEV Ig preparation, diluted 1:10 in PBS. At indicated points in time, serum was collected and TBEV ELISA, NT, and HAI titers were determined. Results for NT and HAI are given as dilutions, and results for ELISA are expressed in Vienna International Units (VIU) as outlined in Materials and Methods. Results are from one of two similar experiments, and values are means of duplicate or quadruplicate determinations with standard deviations below 10%.

a longer period of time after infection (Fig. 3, cf. upper and lower panels).

In vitro ADE of TBEV infection. In vitro ADE of viral infection has been demonstrated in several experimental models and for TBEV as well (17, 27). To allow direct comparison of our in vivo results with those obtained by others in vitro, we tested whether TBEV antibodies as applied in our experimental model would also be able to enhance TBEV replication in vitro. As can be seen (Fig. 5), both the human as well as the murine TBEV antibodies were able to enhance TBEV replication in resident peritoneal macrophages of the strain of mice used in our in vivo experiments.

DISCUSSION

The current study evaluated antibody-mediated passive protection in a mouse model and addressed the question of in vivo ADE of infection with a flavivirus, TBEV. The animal model employed was characterized in detail, and experiments with human, i.e., heterologous, Ig and murine Ig were performed in parallel, with the objective of generating relevant experimental results where clinical studies are hardly feasible.

TBEV caused lethal infection after either s.c. or i.p. infec-

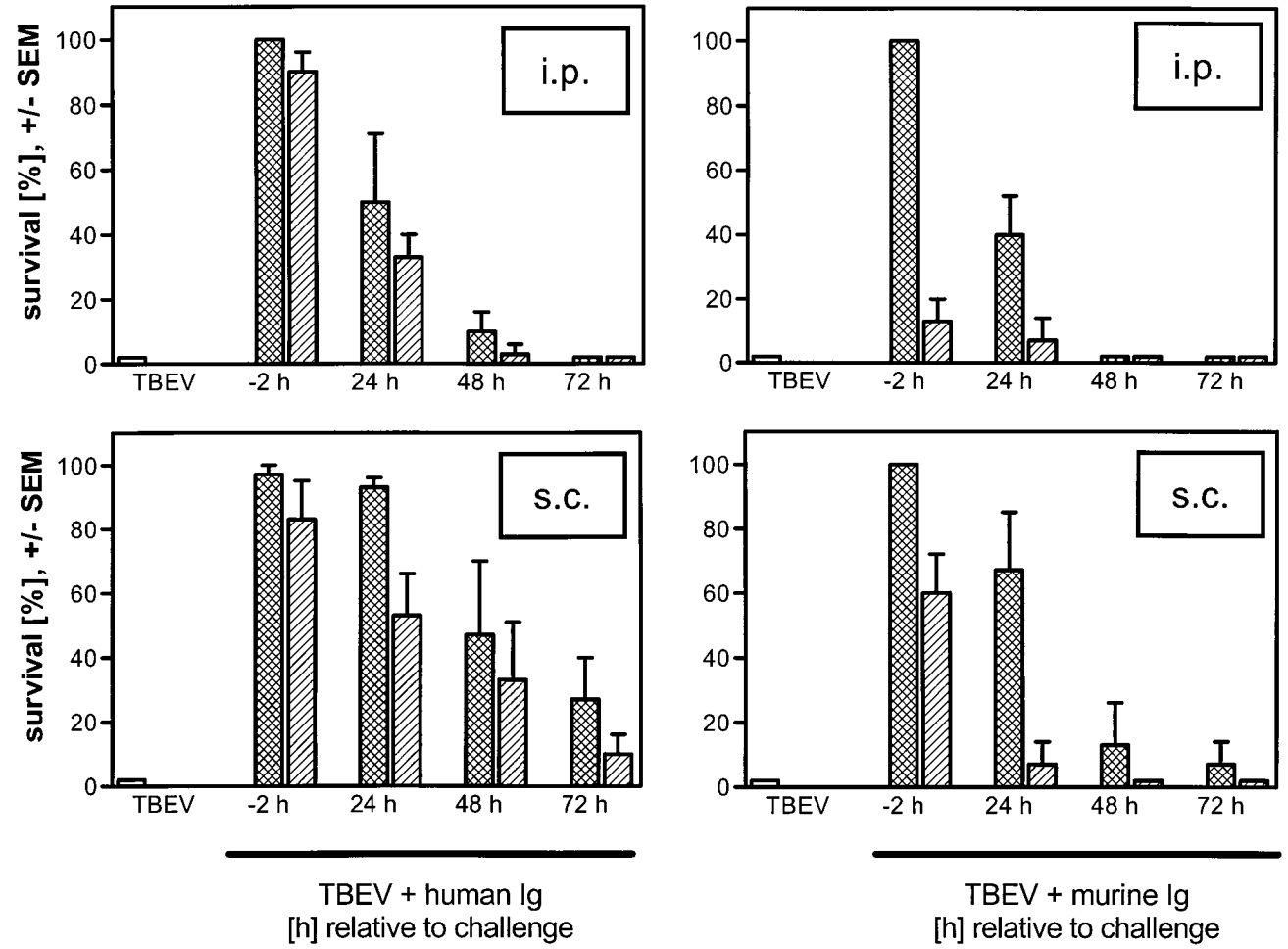

FIG. 3. Mice were infected with 1,000 PFU of TBEV per animal, either i.p. (upper panels) or s.c. (lower panels). At indicated intervals before or after infection, mice were s.c. administered 0.2 ml of TBEV antibodies from either a human (left panels) or a murine (right panels) source, in either a highly protective (cross-hatched bars: 1:10 for human Ig, 1:3 for murine Ig) or a moderately protective (hatched bars: 1:100 for human Ig, 1:30 for murine Ig) dilution in PBS. Mice only infected with TBEV as described and receiving no Ig treatment served as controls (open bars). Survival was monitored for 28 days or until no further deaths occurred for a consecutive week, and results given are means of survival (percent) ± SEMs of three or more experiments (10 animals per group in each experiment).

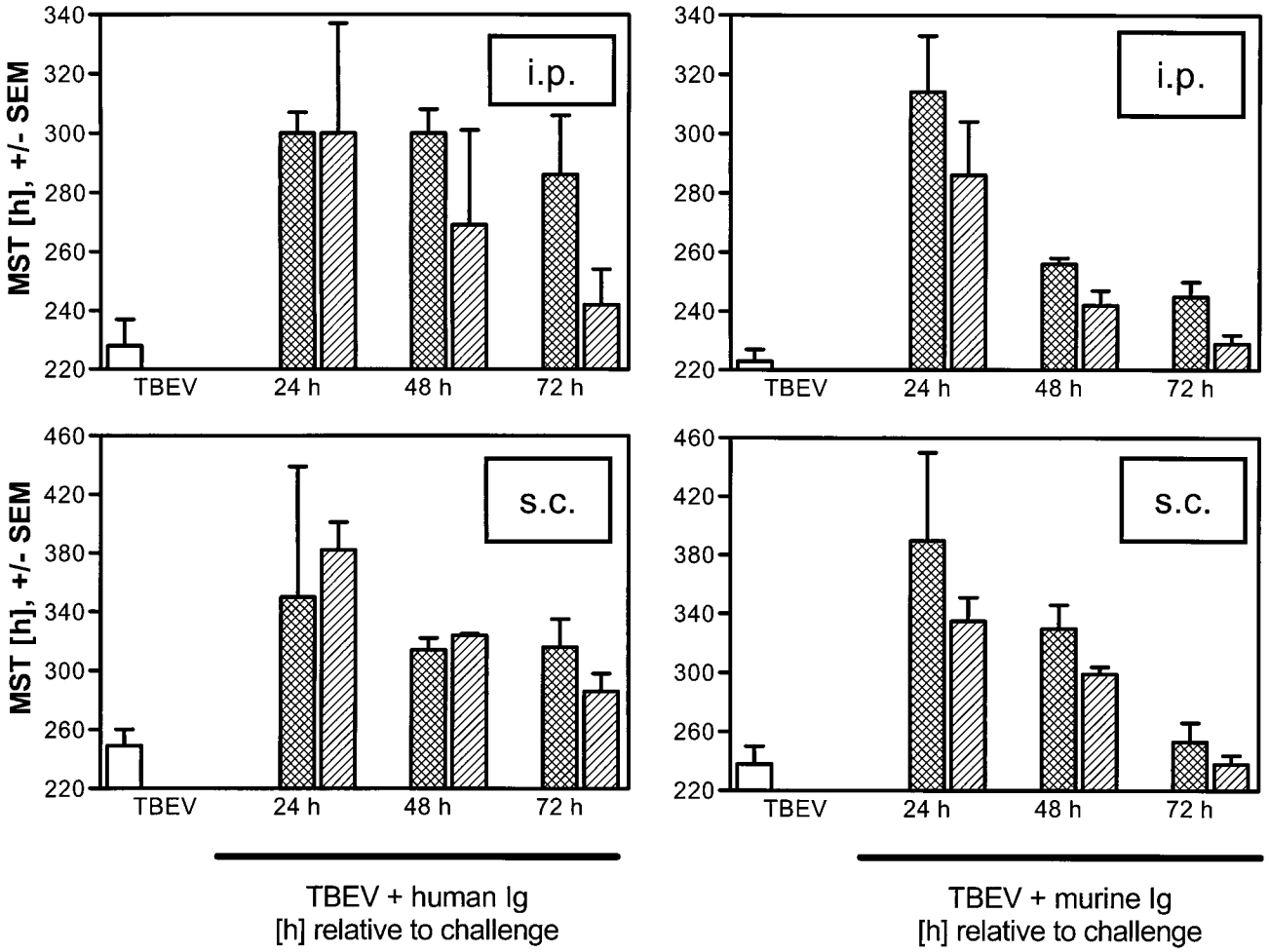

FIG. 4. Mice were treated as for Fig. 3. Results given are MST ± SEMs of animals that died during the observation period, and they are obtained from three or more experiments (10 animals per group in each experiment).

tion of mice. More virus was needed to reach the LD_{50} after s.c. inoculation than after i.p. infection. MST in this murine model of TBEV infection was approximately 10 days after infection with 1,000 PFU of TBEV via either route. Thus, the course of infection is considerably shorter compared to the course of infection in humans. When development of viremia was determined by either plaque titration or sample transfer (Fig. 1), the kinetics were comparable after i.p. and s.c. challenge, i.e., viremia was consistently detectable as early as 24 h after TBEV infection. With respect to establishment of TBEV replication in the brain, plaque titration was not sensitive enough to reveal differences between s.c. or i.p. infection. However, sample transfer demonstrated the onset of TBEV replication in the brain after s.c. infection to be delayed by approximately 1 day compared to i.p. infection (approximately 3 days after i.p. infection versus 4 days after s.c. infection) (Fig. 1). Infection of a few recipients (<50%) before day 3 after i.p. TBEV challenge or day 4 after s.c. TBEV challenge is likely due to minimal virus-bearing blood contamination of the brain transferred, as observed by others (2), rather than TBEV replication, and thus TBEV replication in the brain is not considered to occur earlier than day 3 after i.p. TBEV infection or day 4 after s.c. TBEV infection.

Previous reports on the kinetics of hematogenous spread and replication of TBEV in the brains of mice were not con-

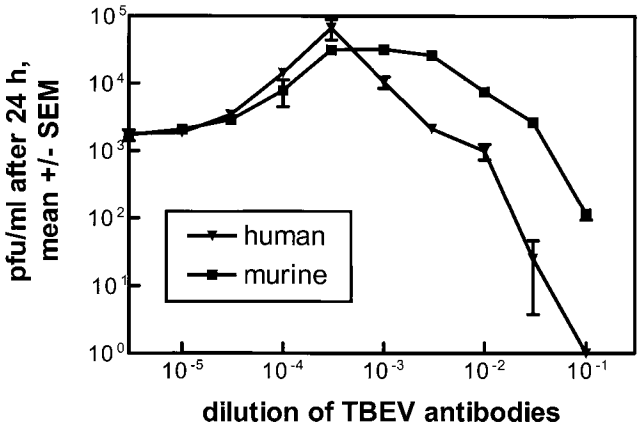

FIG. 5. Macrophages were prepared by adherence to plastic as described in Materials and Methods and, in the presence of the indicated final dilutions of TBEV antibodies, infected with TBEV at 5 PFU per cell for 1 h. Virus and antibodies were subsequently washed away, and the cells were further cultured for 24 h. Supernatants were then collected for determination of TBEV titers by plaque assay. Results are given as PFU of TBEV per ml of supernatant and are means ± SEM of three experiments. The TBEV titers that develop in the absence of antibodies are represented by the point where the curve and the *y* axis meet (2,028 ± 19 PFU/ml).

gruent. As early as 1955, Környey demonstrated that after peripheral TBEV infection of mice, an initial viremia appeared and, simultaneously with initiation of virus replication in the brain, dropped to below detectable levels (18). More recently, Jacobs et al., after applying anti-E immunofluorescence of PS cells after coculture for 7 days with blood or brain specimens of infected animals, demonstrated a viremic phase of 3 days starting from the second day after infection, followed by a drop to zero of blood TBEV titers and a concomitant rise in TBEV titers in the brain (15). Results from plaque titrations of blood or brain samples as done in the current study are in good agreement with both of these observations. On the other hand, the results of Kovac and Moritsch, who by sample transfer detected TBEV in the blood and brain from early after infection up until the death of experimentally infected mice (19), fully concur with sample transfer results of the current study. Based on direct comparison of TBEV detection by plaque assay or sample transfer, we thus propose that differences between earlier reports might reflect the different sensitivity of the experimental procedures applied.

To determine in vivo recovery of human Ig after peripheral inoculation in mice, TBEV ELISA, NT, and HAI titers were measured in murine serum obtained at different times after treatment of mice with human Ig. As can be seen (Fig. 2), the titers reached their maximum at approximately 1 to 2 days after s.c. administration before beginning to decline following similar kinetics. Others have recently shown that, after TBEV vaccination, the ELISA titer is a good surrogate marker for the development of NT and HAI titers of TBEV antibodies (14). The demonstration in our study of closely parallel development of TBEV ELISA, NT, and HAI titers in serum after administration of an Ig preparation with a high TBEV titer is in good agreement with this other observation.

Preexposure prophylaxis by passive administration of either homologous or heterologous antibodies provided an effective means of protection against TBEV-induced lethality (Fig. 3). This is in agreement with the results of earlier studies (11, 13, 25, 28). In extension of these results, postexposure Ig treatment of mice as demonstrated here provided at least partial protection if given within a limited time after infection. Generally, the observed degree of protection was directly related to the amount of antibodies applied, and the degree of postexposure protection was inversely correlated with increasing time between infection and Ig treatment.

As expected, the decline of passive protection by human Ig follows faster kinetics than protection by Ig from a murine source. Given a half-life of human Ig in mice of approximately 10 days and the rather rapid development of TBEV-induced disease in mice—with an MST of only about 10 days after infection—serum clearing of human Ig was unlikely to play an important role in our experimental setting. As to the amounts of the human TBEV Ig preparation used for treatment of mice in our experimental study, they do well correspond to those recommended for a clinical setting. In particular, 0.2 ml of the 1:10- or 1:100-diluted human TBEV Ig preparation as applied to mice would transform to 1.25 or 0.125 ml/kg, respectively (see TBEV antibodies in Materials and Methods).

Interestingly, both murine and human Igs were more potent in protecting mice against s.c. than i.p. TBEV challenge. Furthermore, the decrease in postexposure protection with increasing time interval between infection and Ig treatment was delayed approximately 1 day after s.c. challenge compared to that for i.p. challenge (Fig. 3). This time difference is comparable to the delay in establishment of TBEV replication in the brains of s.c. infected mice (Fig. 1). Thus, these findings indicate that passive protection by Ig is possible only before infection of the brain is established. This idea is further supported by the notion that Ig treatment—even when 10-fold the maximum amount of Ig, as used in the current study, was applied to mice 2 days before infection—could never protect mice against direct intracerebral infection with TBEV (data not shown).

Others, however, have reached different conclusions with different flaviviruses in mouse models: preexposure antibody-mediated protection against intracerebral infection (4, 9) and protection even when antibodies were administered after infection of the brain has been initiated (4, 5) have been reported for yellow fever and West Nile viruses, but also, ADE of yellow fever or Japanese encephalitis virus infection of the mouse by pre- or postexposure treatment with antiviral antibodies have been reported (3, 8). Obviously, the issue is complex. We would nevertheless like to stress that the studies referred to were performed with monoclonal antibodies rather than polyclonal antisera or Ig preparations as applied in our study, and so the conclusions reached by these other studies cannot be compared to ours. Furthermore, it has to be kept in mind that while the cited studies were performed with mosquito-borne viruses, our study with TBEV is the only tick-transmitted member of the flaviviruses that the respective phenomena have been studied with so far.

In humans, use of passive Ig against TBEV infection is recommended up until 96 h postexposure only, as it cannot be ruled out that Ig treatment shortly before onset or during viremia might possibly have a negative effect on disease development (20). Disease development in our model follows much faster kinetics, and thus Ig was certainly applied during viremia and even when infection of the brain was ongoing. Still, in every single experiment where Ig treatment did have a detectable effect, it was always beneficial in terms of improved survival or at least prolonged MST. None of the conditions applied, regardless of the amount or the source of Ig or the time of treatment, allowed for demonstration of an enhancement of TBEV infection in vivo as evidenced by reduced survival or decreased MST. Even at challenge doses as low as 10 PFU per animal representing approximately an LD_{50}, a situation favorable for the demonstration of ADE in vitro, and with Ig dilutions as low as 1:3,000, no indication of ADE in vivo could be found.

The same TBEV antibodies shown to be protective in our in vivo model were, however, well able to enhance TBEV replication in peritoneal macrophages of mice in vitro (Fig. 5), in agreement with earlier studies performed with monoclonal TBEV antibodies (27) or murine TBEV-immune serum (17). In extension of these results, the current study demonstrates that human TBEV Ig is also capable of enhancing TBEV replication in murine macrophages. In particular, ADE by human TBEV Ig was highest at a dilution of 1:3,000 (25- to 40-fold increase), and for murine TBEV antibodies ADE was highest at 1:1,000 (15- to 17-fold increase). ADE is known to depend on binding of antibodies to Fc receptors (26), and murine Fc receptors bind both murine and human Ig (29), allowing for ADE of TBEV infection in mouse macrophages in vitro.

Neither sublethal challenge doses of TBEV nor suboptimal Ig dilutions, even when applied together, could thus provide any indication of ADE occurring in vivo, despite demonstrable ADE with the same combinations in vitro. One further experimental setting favorable to the demonstration of ADE would have been the use of antibodies specific for an antigenically related virus instead of antibodies raised against the challenge virus itself. In contrast to the dengue viruses, however, where types 1 to 4 share only between 62 and 77% amino acid ho-

mology within their E proteins (23) and ADE by type-cross-reactive antibodies has been implicated in the pathogenesis of dengue hemorrhagic fever-dengue shock syndrome, the different strains of TBEV share 96% or more amino acid homology within their E protein (23).

In vitro ADE has been described for viruses other than those of the flavivirus genus, such as human immunodeficiency virus, for example (30). Whether the phenomenon occurs in vivo and whether it relates to the clinical outcome of infection with the virus remain unresolved (24), but even the possibility is of concern especially in relation to vaccine safety (7). Based on our results, however, one may question whether in vitro ADE reflects and should be taken as a correlate for the in vivo situation.

In conclusion, passive protection against TBEV infection is well demonstrable by TBEV Ig, and the protection conferred is directly related to the amounts of Ig applied. For postexposure prophylaxis, protection is inversely related to the length of the interval between infection and Ig treatment. With all of the experimental treatments applied in the current study, in vivo enhancement of TBEV infection could not be demonstrated, despite demonstration of ADE in vitro by the same combinations of TBEV antibodies and virus.

ACKNOWLEDGMENTS

We thank I. Burger and E. Attakpah for excellent animal experimentation, M. Bachmann for skillful help with in vitro enhancement assays, M. Pfundner for secretarial assistance (IMMUNO AG, Vienna), P. N. Barrett for fruitful discussions and for kindly donating virus, and O. Enzersberger and F. Dorner for providing HAI and NT testing of TBEV antibodies (Biomedical Research Center, IMMUNO AG, Orth/Donau).

We thank IMMUNO AG (Vienna, Austria) for financial support.

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